

MULTIPLY-SUBSTITUTED PROTEASE VARIANTS

5 **Background of the Invention**

Serine proteases are a subgroup of carbonyl hydrolases. They comprise a diverse class of enzymes having a wide range of specificities and biological functions. Stroud, R. Sci. Amer., **131**:74-88. Despite their functional diversity, the catalytic machinery of serine proteases has been approached by at least two genetically distinct families of enzymes: 1) the subtilisins and 2) the mammalian chymotrypsin-related and homologous bacterial serine proteases (e.g., trypsin and *S. gresius* trypsin). These two families of serine proteases show remarkably similar mechanisms of catalysis. Kraut, J. (1977), Annu. Rev. Biochem., **46**:331-358. Furthermore, although the primary structure is unrelated, the tertiary structure of these two enzyme families bring together a conserved catalytic triad of amino acids consisting of serine, histidine and aspartate.

Subtilisins are serine proteases (approx. MW 27,500) which are secreted in large amounts from a wide variety of *Bacillus* species and other microorganisms. The protein sequence of subtilisin has been determined from at least nine different species of *Bacillus*. Markland, F.S., et al. (1983), Hoppe-Seyler's Z. Physiol. Chem., **364**:1537-1540. The three-dimensional crystallographic structure of subtilisins from *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and several natural variants of *B. lentus* have been reported. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972), Biochemistry, **11**:2439-2449) or product complexes (Robertus, J.D., et al. (1976), J. Biol. Chem., **251**:1097-1103) have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisin; Svendsen, B. (1976), Carlsberg Res. Commun., **41**:237-291; Markland, F.S. Id.) as well as at least one report wherein the side chain of methionine at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer, D.C., et al. (1965), J. Biol. Chem., **244**:5333-5338) and extensive site-specific mutagenesis has been carried out (Wells and Estell (1988) TIBS **13**:291-297)

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Summary of the Invention

It is an object herein to provide a protease variant containing a substitution of an amino acid at one or more residue positions equivalent to residue positions selected from the group consisting of 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90,
5 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273 of *Bacillus amyloliquefaciens* subtilisin.

A protease variant is described comprising an amino acid sequence having a substitution at one or more residue positions equivalent to residue positions selected from the group consisting of 7, 23, 26, 28, 29, 30, 31, 47, 66, 69, 73, 82, 85, 88, 90,
10 92, 93, 105, 113, 139, 148, 149, 150, 151, 178, 200, 201, 231, 233, 267 and 273 of *Bacillus amyloliquefaciens* subtilisin. The protease variant of claim includes at least one improved property selected from improved a) wash performance and b) stability as compared to the wild type. In one embodiment, the protease to which these variants is compared is the wild-type GG36 (SEQ ID. NO.6). The improved stability can be
15 improved thermostability.

The protease variants can be selected from at least one position equivalent to 7N, 23A, 26S, 26T, 28C, 28G, 28S, 28T, 29G, 30A, 31A, 31I, 31T, 31V, 47D, 65M, 66D, 66E, 73G, 73T, 82R, 85D, 85G, 85S, 85L, 85V, 85Y, 88S, 90A, 90I, 90M, 92E, 92R, 93A, 93G, 93S, 93T, 105D, 105E, 105G, 105R, 113D, 139A, 148G,
20 149A, 149F, 149G, 149H, 149S, 149W, 150A, 150C, 150F, 150L, 151V, 178S, 178C, 178L, 201C, 231G, 231S, 233G, 233V, 267R, 267I, 273S of *Bacillus amyloliquefaciens* subtilisin.

The protease variant having improved wash performance at about 20 degrees
25 centigrade, at a concentration of 0.5 to 1.0 ppm protease and at water hardness conditions of about 3 grains per gallon mixed Ca²⁺/Mg²⁺ hardness (Japanese wash conditions) comprises a substitution of at least one residue equivalent to 31, 47, 85, 90, 92, 105, 113, 148, 149, 151, 174, 200 and 201 of *Bacillus amyloliquefaciens*. The substitutions are selected from the group consisting of 31I, 31V, 47S, 47D, 85G, 90V, 92E, 105D,
30 105E, 113D, 148W, 151V, 174G, 174S, 200S and 201C.

The protease variant can also have improved wash performance at about 40 degrees centigrade, at a protease concentration of 0.3-0.5 ppm protease and at

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water hardness conditions of about 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness. The protease variant of having improved wash performance under these conditions comprises a substitution at one or more positions equivalent to 31, 69, 82, 148, 201, 203, 231, 233, 258, 267 and 270 of *Bacillus amyloliquefaciens* subtilisin.

5 These protease variants can comprise at least one substitution at one or more positions equivalent to 31, 69, 82, 148, 201, 231, 233 and 267 of *Bacillus amyloliquefaciens* subtilisin is selected from the group of 31I, 31V, 69G, 82R, 148G, 201S, 231V, 233G and 267R.

10 The protease variant of claim 1, wherein said variant has improved wash performance at about 10 degrees to about 30 degrees centigrade, at a concentration of 1.0 ppm protease and at water hardness conditions of about 6 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness (North American conditions). These protease variants comprise a substitution at one or more positions equivalent to 61,
15 66, 105, 203 and 258 of *Bacillus amyloliquefaciens* subtilisin. These at least one substitution at one or more positions equivalent to 61, 66, 105, 203, 216 and 258 of *Bacillus amyloliquefaciens* subtilisin can be selected from the group of 61E, 66D, 105D, 105E, 203D, 203E, 216E and 258E.

20 It is a further object to provide DNA sequences encoding such protease variants, as well as expression vectors containing such variant DNA sequences.

Still further, another object of the invention is to provide host cells transformed with such vectors.

25 There is further provided a cleaning composition comprising a protease variant of the present invention.

Additionally, there is provided an animal feed comprising a protease variant of the present invention.

Also provided is a composition for the treatment of a textile comprising a protease variant of the present invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A-C depict the DNA (SEQ ID NO:1) and amino acid sequences (SEQ ID NO:2) for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene.

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Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (SEQ ID NO:3). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (SEQ ID NO:4). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (SEQ ID NO:5). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276) (SEQ ID NO:6). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig 4 depicts the pVS08 B. subtilis expression vector.

Fig. 5 depicts the orientation of the forward Apal primer, the reverse Apal primer, the reverse mutagenic primer, and the forward mutagenic primer.

Detailed Description of the Invention

Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include α -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxyl-proteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

The present invention includes protease enzymes which are non-naturally occurring carbonyl hydrolase variants (protease variants) having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. Specifically, such protease variants have an amino acid sequence not found in nature, which is derived by substitution of a plurality of amino acid residues of a precursor protease with different amino acids. The precursor protease may be a naturally-occurring protease or a recombinant protease.

The protease variants useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application reference is made to various amino acids by way of common one - and three-letter codes. Such codes are identified in Dale, M.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

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The protease variants useful herein are preferably derived from a *Bacillus* subtilisin. More preferably, the protease variants are derived from *Bacillus amyloliquefaciens*, *Bacillus lentus* subtilisin and/or subtilisin 309.

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide
5 bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares
10 a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related
15 proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin
20 sequence presented in Figure 1.

"Recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods
25 to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent RE 34,606, US Patent 5,204,015 and US Patent 5,185,258, U.S. Patent 5,700,676, U.S. Patent 5,801,038, and U.S. Patent 5,763,257.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include
30 gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

A "protease variant" has an amino acid sequence which is derived from the amino
35 acid sequence of a "precursor protease". The precursor proteases include naturally-

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occurring proteases and recombinant proteases. The amino acid sequence of the protease variant is "derived" from the precursor protease amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid
 5 sequence of the precursor protease rather than manipulation of the precursor protease enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

10 Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 5, 6, 7, 8, 12, 23, 24, 26, 28-31, 34, 38, 43, 47, 50, 52, 57, 63, 65, 66, 69, 70, 72, 73, 73, 82 - 85, 86, 88, 89, 90, 92, 93, 99, 103, 105, 113, 114, 116, 117, 119, 121, 125, 136, 138, 139, 142, 145, 147-151, 172, 174, 176, 177, 178, 179, 193, 196, 198, 199, 200, 201, 202, 203, 204, 206, 207, 218, 219, 220,
 15 223, 228, 229, 231, 232, 233, 250, 252, 258, 263, 264, 266, 267, 270 and 273 of *Bacillus amyloliquefaciens* subtilisin are identified herein.

Specific substitutions of amino acids at one or more residue positions equivalent to A1E, A1D, A1R, A1K, W6R, G7N, Q12H, G23A, F24S, V26S, V26T, V28C, V28S, V28T, A29G, V30A, L31A, L31I, L31T, L31V, T38S, N43D, G47D, G47S, L50F, G52E, T57A,
 20 G65M, T66D, T66E, G69_, I72C, I72L, I72V, A73L, A73G, A73T, A73V, L82R, A85D, A85G, A85L, A85S, A85V, A85Y, P86D, A88S, E89G, L90A, L90I, L90M, L90V, A92E, A92R, V93A, V93G, V93I, V93S, V93T, S99G, S103C, S105D, S105E, S105G, S105R, W113D, A114C, A114G, A114S, A114T, N116D, N117S, M119A, M119C, M119F, M119G, M119S, M119T, M119V, H120R, Q121I, G127A, S128D, S128L, E136R, V139A, A142E,
 25 R145G, V147C, V147G, V147L, V147S, L148G, L148W, V149A, V149F, V149G, V149H, V149S, V149W, V150A, V150C, V150F, V150L, A151V, S156E, S156D, A169G, R170M, A172T, A174G, A174S, A174T, G178C, G178L, G178S, I198A, I198L, I198M, I198V, I198T, M199V, A200S, P201C, P201S, V203R, V203D, V203E, V203L, V203S, N204D, Q206R, S216D, N218S, S216E, S216R, A231G, A231S, A232C, A232G, A232I, A232L,
 30 A232M, A232N, A231V, A232T, A232V, A232S, L233G, L233V, I246M, I246V, R247C, N252S, S256G, T253D, T253E, T253K, T253R, G258D, G258E, G258K, G258R, Y263H, G264S, L267I, L267R, A270L, A270V, A273S, T260A in *Bacillus lentus* (using BPN' numbering). Specific combinations of amino acids having at least the combinations V26S / N218S; G69 / Q12R; L90V / N204D; V93A / S103C; V93T / E136G; V139A / V150A; A142E
 35 / E89G; L148G / F24S; V149S / Q12H; V150A / T38S; V150C / N218S; A174G / N204D;

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A174S / G52E / A172T; G178C / N43D; I198M / V93I; I198V / V30A; A200S / N204D; P201S / L50F; P201S / T57A; A231G / M119V; A232I / A108V; A231V / Q206R; A232M / N116D; A232N / I16D; G264S / R145G; L267I / Y263H; L267R / S99G; L267R / N252S; A270V / E136R; and A172T / A174S / G52E in *Bacillus lentus* (using BPN' numbering).

5 Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 14, 31, 61, 82, 92, 203, 233, 253, 258, 267 and 270 of *Bacillus amyloliquefaciens* subtilisin are identified herein as providing improved wash performance under European wash conditions. Specific substitutions of amino acids at one or more residue positions corresponding to these positions are
10 described in the Examples.

 Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 31, 47, 61, 66, 85, 86, 88, 92, 105, 113, 148, 149, 151, 201, 203, 216, 253, and 258 of *Bacillus amyloliquefaciens* subtilisin are identified herein as providing improved wash performance under Japanese
15 wash conditions.

 Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 61, 66, 105, 203, 216 and 258 of *Bacillus amyloliquefaciens* subtilisin are identified herein as providing improved wash performance under North American conditions.

20 Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 7, 8, 23, 26, 28-31, 65, 70, 72, 73, 85, 86, 88, 90, 93, 114, 119, 147-150, 177, 178, 198, 203, 228, 231, 232, 246 and 273 of *Bacillus amyloliquefaciens* subtilisin are identified herein as providing improved thermostability under European wash conditions..

25 These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions are made
30 at the equivalent amino acid residue positions in *B. lentus* corresponding to those listed above.

 A residue (amino acid) position of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position
35 in either primary or tertiary structure) or analogous to a specific residue or portion of that

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residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 98%, 95%, 90%, 85%, 80%, 75%, 70%, 50% or at least 45% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained. Siezen et al. (1991) Protein Eng. 4(7):719-737 shows the alignment of a large number of serine proteases. Siezen et al. refer to the grouping as subtilases or subtilisin-like serine proteases.

For example, in Fig. 3, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (carlsbergensis) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

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“Equivalent residues” may also be defined by determining homology at the level of tertiary structure for a precursor protease whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R\ factor = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

Equivalent residues which are functionally similar to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the substitution of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such substitutions should not result in a naturally-occurring sequence. The protease variants of the present invention include the mature forms of protease variants, as

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well as the pro- and prepro-forms of such protease variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the protease variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion
5 of the mature form of a protease which when removed results in the appearance of the "mature" form of the protease. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing protease variants is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other protease
10 prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protease or to the N-terminal portion of a proprotease which may participate in the secretion of the mature or pro forms of the protease. This definition of signal sequence is a functional one, meant to include all those amino acid sequences
15 encoded by the N-terminal portion of the protease gene which participate in the effectuation of the secretion of protease under native conditions. The present invention utilizes such sequences to effect the secretion of the protease variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from
20 *Bacillus lentus* (ATCC 21536).

A "prepro" form of a protease variant consists of the mature form of the protease having a prosequence operably linked to the amino terminus of the protease and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is
25 operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic
30 insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which
35 serve equivalent functions and which are, or become, known in the art.

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The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent RE 34,606 and/or 5,441,882 to render them incapable of secreting enzymatically active endoprotease. A host cell useful for expressing protease is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protease include *Bacillus subtilis* I168 (also described in US Patent RE 34,606; US Patent 5,264,366; and 5,441,882, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus*-strain such as *B. licheniformis*, *B. lentus*, etc. A particularly useful host cell is the *Bacillus* strain BG2864. The construction of strain BG2864 is described in detail in D. Naki, C. Paech, G. Ganshaw, V. Schellenberger. Appl Microbiol Biotechnol (1998) 49:290-294.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the protease variants or expressing the desired protease variant. In the case of vectors which encode the pre- or prepro-form of the protease variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked," when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor protease may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protease of interest, preparing genomic libraries from organisms expressing the protease, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The cloned protease is then used to transform a host cell in order to express the protease. The protease gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, i.e., transcribed, by the

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host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the protease gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the protease gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the protease gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

The gene can be a natural *B. lentus* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor protease may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor protease is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor protease. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protease gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protease. Such modifications include the production of recombinant proteases as disclosed in US Patent RE 34,606 and EPO Publication No. 0 251 446 and the production of protease variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protease variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protease is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protease gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protease gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites

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are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant enzymes of the present invention may have other modified properties such as K_m , k_{cat} , k_{cat}/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present, for example, in the preparation of peptides or for hydrolytic processes such as laundry uses.

Stability, for example thermostability, is an aspect which could be accomplished by the protease variant described in the examples. The stability may be enhanced or diminished as is desired for various uses. Enhanced stability could be effected by substitution one or more residues identified in the present application and, optionally, substituting another amino acid residue not one of the same. Thermostability is maintaining enzymatic activity over time at a given temperature. An improved thermostability involves the maintenance of a greater amount of enzymatic activity by the variant as compared to the precursor protease. For example, an increased level of enzymatic activity of the variant as compared to the precursor at a given temperature, typically the operation temperature of as measured.

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In one aspect of the invention, the objective is to secure a variant protease having altered, preferably improved wash performance as compared to a precursor protease in at least one detergent formulation and or under at least one set of wash conditions.

There is a variety of wash conditions including varying detergent formulations, wash
5 water volume, wash water temperature and length of wash time that a protease variant might be exposed to. For example, detergent formulations used in different areas have different concentrations of their relevant components present in the wash water. For example, a European detergent typically has about 3000-8000 ppm of detergent components in the wash water while a Japanese detergent typically has less than 800, for
10 example 667 ppm of detergent components in the wash water. In North America, particularly the United States, a detergent typically has about 800 to 2000, for example 975 ppm of detergent components present in the wash water.

A low detergent concentration system includes detergents where less than about 800 ppm of detergent components are present in the wash water. Japanese detergents are
15 typically considered low detergent concentration system as they have approximately 667 ppm of detergent components present in the wash water.

A medium detergent concentration includes detergents where between about 800 ppm and about 2000ppm of detergent components are present in the wash water. North American detergents are generally considered to be medium detergent concentration
20 systems as they have approximately 975 ppm of detergent components present in the wash water. Brazil typically has approximately 1500 ppm of detergent components present in the wash water.

A high detergent concentration system includes detergents where greater than about 2000 ppm of detergent components are present in the wash water. European
25 detergents are generally considered to be high detergent concentration systems as they have approximately 3000-8000 ppm of detergent components in the wash water.

Latin American detergents are generally high suds phosphate builder detergents and the range of detergents used in Latin America can fall in both the medium and high detergent concentrations as they range from 1500 ppm to 6000 ppm of detergent
30 components in the wash water. As mentioned above, Brazil typically has approximately 1500 ppm of detergent components present in the wash water. However, other high suds phosphate builder detergent geographies, not limited to other Latin American countries, may have high detergent concentration systems up to about 6000 ppm of detergent components present in the wash water.

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In light of the foregoing, it is evident that concentrations of detergent compositions in typical wash solutions throughout the world varies from less than about 800 ppm of detergent composition ("low detergent concentration geographies"), for example about 667 ppm in Japan, to between about 800 ppm to about 2000 ppm ("medium detergent concentration geographies"), for example about 975 ppm in U.S. and about 1500 ppm in Brazil, to greater than about 2000 ppm ("high detergent concentration geographies"), for example about 3000 ppm to about 8000 ppm in Europe and about 6000 ppm in high suds phosphate builder geographies.

The concentrations of the typical wash solutions are determined empirically. For example, in the U.S., a typical washing machine holds a volume of about 64.4 L of wash solution. Accordingly, in order to obtain a concentration of about 975 ppm of detergent within the wash solution about 62.79 g of detergent composition must be added to the 64.4 L of wash solution. This amount is the typical amount measured into the wash water by the consumer using the measuring cup provided with the detergent.

As a further example, different geographies use different wash temperatures. The temperature of the wash water in Japan is typically less than that used in Europe. For example, the temperature of the wash water in North America and Japan can be between 10 and 30 degrees centigrade, for example about 20 degrees C, whereas the temperature of wash water in Europe is typically between 30 and 50 degrees centigrade, for example about 40 degrees C.

As a further example, different geographies may have different water hardness. Water hardness is typically described as grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$. Hardness is a measure of the amount of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the water. Most water in the United States is hard, but the degree of hardness varies. Moderately hard (60-120 ppm) to hard (121-181 ppm) water has 60 to 181 parts per million [parts per million converted to grains per U.S. gallon is ppm # divided by 17.1 equals grains per gallon] of hardness minerals.

Water	Grains per gallon	Parts per million
Soft	less than 1.0	less than 17
Slightly hard	1.0 to 3.5	17 to 60
Moderately hard	3.5 to 7.0	60 to 120
Hard	7.0 to 10.5	120 to 180
Very hard	greater than 10.5	greater than 180

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European water hardness is typically greater than 10.5 (for example 10.5-20.0) grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$, for example about 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$. North American water hardness is typically greater than Japanese water hardness, but less than European water hardness. For example, North American water hardness can be between 3 to 10 grains, 3-8 grains or about 6 grains. Japanese water hardness is typically the lower than North American water hardness, typically less than 4, for example 3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$.

Accordingly one aspect of the present invention includes a protease variant that shows improved wash performance in at least one set of wash conditions. Another aspect of the present invention includes a protease variant that shows improved wash performance in at least two sets of wash conditions.

In another aspect of the invention, it has been determined that modification at one or more residue positions, for example by substitution, insertion or deletion of an amino acid equivalent to residue positions selected from the group consisting of 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 86, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 203, 207, 219, 220, 223, 229, 233, 250, 258, 266, 267, 270 and 273 of *Bacillus amyloliquefaciens* subtilisin are important in improving the wash performance of the enzyme. The amino acids substituted, inserted or deleted contemplated by the inventors include, but are not limited to alanine (Ala or A), arginine (Arg or R), aspartic acid (Asp or D), asparagines (Asn or N), cysteine (Cys or C), glutamic acid (Glu or E), glutamine (Gln or Q), glycine (Gly or G), histidine (His or H), isoleucine (Iso or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophane (Trp or W), tyrosine (Tyr or Y) and/or valine (Val or V).

One aspect of the present invention includes a protease variant further comprising at least one additional replaced amino acid at one or more residue positions equivalent to residue positions or selected from the group consisting of 6, 9, 11-12, 19, 25, 37-38, 54-59, 68, 71, 89, 111, 115, 120, 121-122, 140, 175, 180, 182, 186, 187, 191, 194, 195, 226 234-238, 241, 260-262, 265, 268, 75, 129, 131, 136, 159, 164, 165, 167, 170, 171, 194, 195, 27, 36, 57, 76, 97, 101, 104, 120, 123, 206, 218, 222, 224, 235, 274, 2, 3, 4, 10, 15, 17, 20, 40, 44, 51, 52, 60, 91, 108, 112, 133, 134, 143, 144, 145, 146, 173, 211, 212, 239, 240, 242, 243, 245, 252, 255, 257, 259, 263, 269, 183, 184, 185, 192, 209, 210, 18, 117, 137, and 244 of *Bacillus amyloliquefaciens*. Specific residues contemplated by the inventors include those equivalent to : I122A, Y195E, M222A, M222S, Y167A, R170S, A194P, D36, N76D,

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H120D, G195E, and K235N of *Bacillus amyloliquefaciens*, which variant is derived from a *Bacillus subtilisin*. Those skilled in the art will recognize the protease variants having these modifications can be made and are described in US Patents 5,741,694; 6,190,900; and 6,197,567, expressly incorporated by reference herein.

5 Still another aspect of the present invention includes a protease variant further comprising at least one additional replaced amino acid at one or more equivalent residue positions from the group consisting of 12, 271, 204, 103, 136, 150, 89, 24, 38, 218, 52, 172, 43, 93, 30, 50, 57, 119, 108, 206, 16, 145, 263, 99, 252, 136, 32, 155, 104, 222, 166, 64, 33, 169, 189, 217, 157, 156, 152, 21, 22, 24, 36, 77, 87, 94, 95, 96, 110, 197, 204, 107, 170, 171, 172, 10 213, 67, 135, 97, 126, 127, 128, 129, 214, 215, 50, 124, 123 or 274 of *Bacillus amyloliquefaciens*. Specific residues contemplated by the inventors include: Y217L, K27R, V104Y, N123S, T274A, N76D, S103A, V104I, S101G, S103A, V104I, G159D, A232V, Q236H, Q245R, N248D, N252K M50, M124 and M222S. Additional specific residues contemplated by the inventors include those equivalent to : Q12R, E271G, N204D, S103C, E136G, V150A, E89G, 15 F24S, T38S, N218S, G52E, A172T, N43D, V93I, V30A, L50F, T57A, M119V, A108V, Q206R, I16D, R145G, Y263H, S99G, N252S, Q136R of *Bacillus amyloliquefaciens*. Protease variants, recombinant DNA encoding mutants at these positions and/or methods for making these modifications are described in US patent Nos. RE 34,606; 5,972,682; 5,185,258; 5,310,675; 5,316,941; 5,801,038; 5,972,682, 5,955,340 and 5,700,676, expressly incorporated by reference 20 herein. In addition, these modifications can also be made using direct *Bacillus* transformation methods as described in Provisional Application Ser. No. 60/423,087 (filed November 1, 2002; Neelam Amin and Volker Schellenberger). In one embodiment, the modifications were performed using fusion PCR techniques (Teplyakov, AV, et al, Protein Eng., 1992 Jul 5(5):413-20). Provisional Application Serial Number __/_____, filed concurrently this date (Chris 25 Leeflang, et al.)

These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protease.

Based on the screening results obtained with the variant proteases, the noted mutations in *Bacillus amyloliquefaciens* subtilisin and their equivalent in *Bacillus lentus* are 30 important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protease variants of the invention are useful in formulating various detergent compositions or personal care formulations such as shampoos or lotions. A number of known compounds are suitable surfactants useful in compositions comprising the 35 protease mutants of the invention. These include nonionic, anionic, cationic, or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri

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Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protease variants of the present invention
5 may be used for any purpose that native or wild-type proteases are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise enhanced performance in a detergent composition (as
10 compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about 0.01 to about 5% (preferably
15 0.1% to 0.5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable
20 for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described protease's denaturing temperature. In addition, proteases of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The present invention also relates to cleaning compositions containing the protease
25 variants of the invention. The cleaning compositions may additionally contain additives which are commonly used in cleaning compositions. These can be selected from, but not limited to, bleaches, surfactants, builders, enzymes and bleach catalysts. It would be readily apparent to one of ordinary skill in the art what additives are suitable for inclusion into the compositions. The list provided herein is by no means exhaustive and should be
30 only taken as examples of suitable additives. It will also be readily apparent to one of ordinary skill in the art to only use those additives which are compatible with the enzymes and other components in the composition, for example, surfactant.

When present, the amount of additive present in the cleaning composition is from about 0.01% to about 99.9%, preferably about 1% to about 95%, more preferably about 1%
35 to about 80%.

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The variant proteases of the present invention can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteases of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

All publications and patents referenced herein are hereby incorporated by reference in their entirety.

Example 1

A large number of protease variants can be produced and purified using methods well known in the art. Mutations can be made in *Bacillus amyloliquefaciens* (BPN') subtilisin or *Bacillus lentus* GG36 subtilisin. The variants can be selected from the following: 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273

Example 2

A large number of the protease variants produced in Example 1 can be tested for performance in two types of detergent and wash conditions using a microswatch assay described in "An improved method of assaying for a preferred enzyme and/or preferred detergent composition", U.S. Serial No. 60/068,796.

The variant proteases can be assayed and tested various detergents. For example, a possible detergent can be 0.67 g/l filtered Ariel Ultra (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme used in each well at 20°C. Another exemplary detergent can be 3.38 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme used in each well at 40°C. A higher relative value as compared to the wild-type could indicate and improve detergent efficacy.

Example 3

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Table 6 lists the variant proteases assayed from Example 1 and the results of testing in four different detergents. The same performance tests as in Example 2 were done on the noted variant proteases with the following detergents. For column A, the detergent was 0.67 g/l filtered Ariel Ultra (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column B, the detergent was 3.38 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme was used in each well at 40°C. For column C, 3.5 ml/l HSP1 detergent (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 8 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column D, 1.5 ml/l Tide KT detergent (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme was used in each well at 20°C.

Example 4

A large number of protease variants were produced and purified using methods well known in the art. All mutations were made in *Bacillus lentus* GG36 subtilisin. The variants are shown in Table 1.

To construct the GG36 site saturated libraries and site specific variants, three PCR reactions were performed: two PCR's to introduce the mutated codon of interest in GG36 and a fusion PCR to construct the expression vector including the desired mutation(s).

The GG36 codons of interest are numbered according to the BPN' numbering (listed in Figures 1 A – C and 3A-B).

For the site saturated library construction:

The method of mutagenesis was based on the region-specific mutation approach (Tepliyakov *et al.*, 1992) in which the creation of all possible mutations at a time in a specific DNA codon was performed using a forward and reversed complimentary oligonucleotide primer set with a length of 30-40 nucleotides enclosing a specific designed triple DNA sequence NNS ((A,C,T or G), (A,C,T or G), (C or G)) that correspond with the sequence of the codon to be mutated and guarantees randomly incorporation of nucleotides at that codon.

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For the site specific variant construction:

The forward and reverse mutagenic primer enclose the desired mutation(s) in the middle of the primer with ~15 bases of homologues sequence on both sides.

- 5 These mutation(s), which cover the codon of interest, are specific for the desired amino acid and are synthesized by design.

The second primer set used to construct the libraries and variants contains the pVS08 *Apal* digestion site together with its flanking nucleotide sequence.

10

Apal primers:

Forward *Apal* primer:

GTGTGTGGGCCCATCAGTCTGACGACC

15

Reverse *Apal* primer:

GTGTGTGGGCCCTATTCGGATATTGAG

- 20 The introduction of the mutation(s) in GG36 molecules was performed using Invitrogen (Carlsbad, CA, USA) Platinum[®] *Taq* DNA Polymerase High Fidelity (Cat. no. 11304-102) together with pVS08 template DNA and Forward mutagenic primer and Reverse *Apal* primer for reaction 1, or Reverse mutagenic primer and Forward *Apal* primer for reaction 2.

- 25 The construction of the expression vector including the desired mutation(s) was accomplished by a fusion PCR using PCR fragment of both reaction 1 and 2, forward and reverse *Apal* primer and Invitrogen Platinum[®] *Taq* DNA Polymerase High Fidelity (Cat. no. 11304-102).

- 30 All PCR's were executed according to Invitrogen protocol supplied with the polymerases, except for the number of cycles: 20 instead of 30. Two separate PCR reactions are performed using Invitrogen Platinum[®] *Taq* DNA Polymerase High Fidelity (Cat. no. 11304-102):

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The amplified linear 5.6 Kb fragment was purified (using Qiagen® Qiaquick PCR purification kit Cat. no. 28106) and digested with *Apal* restriction enzyme to create cohesive ends on both sides of the fusion fragment:

- 35 µL purified DNA fragment

5 - 4 µL React® 4 buffer (Invitrogen®: 20 mM Tris-HCl, 5 mM MgCl₂, 50 mM KCl, pH 7.4)

- 1 µL *Apal*, 10 units/ml (Invitrogen® Cat. no. 15440-019)

Reaction conditions: 1 hour, 30°C.

10 An additional digestion with Invitrogen *DpnI* was performed to remove the pVS08 template DNA:

- 40 µL *Apal* digested DNA fragment

- 1 µL *DpnI*, 4 units/µL (Invitrogen® Cat. no. 15242-019)

Reaction conditions: 16-20 hours, 37°C.

15

Ligation of the double digested and purified fragment results in new circular DNA containing the desired mutation with was directly transformed to competent *Bacillus subtilis* :

- 30 µL of purified *Apal* and *DpnI* digested DNA fragment

- 8 µL T4 DNA Ligase buffer (Invitrogen® Cat. no. 46300-018)

20 - 1 µL T4 DNA Ligase, 1 unit/µL (Invitrogen® Cat. no. 15224-017)

Reaction conditions: 16-20 hours, 16°C.

Ligation mixtures were transformed to *Bacillus subtilis* BG2864 (Naki *et al.*, 1998) using the method of Anagnostopoulos and Spizizen (1961) and selected for
25 chloramphenicol resistance and protease activity.

Method for protein production

Inoculated 1-50 µL of glycerol culture in Mops media (Frederick C. Neidhardt *et al.*,
30 1974) containing carbon source (Glucose and Maltodextrine, 10.5 and 17.5 g/l) a nitrogen source (Urea, 3.6 g/l), and essential nutrients such as phosphate (0.5 g/l) and sulphate (0.5 g/l) and further supplemented with trace elements (Fe, Mn, Zn, Cu, Co, 1-4 mg/ml). The medium was buffered with a MOPS/Tricine mixture resulting in a pH varying 7 to 8. Incubate the culture for 1-5 days at 37°C/220 rpm (Infors HT® Multitron II).

References:

- Protein engineering of the high-alkaline serine protease PB92 from Bacillus alcalophilus: functional and structural consequences of mutation at the S4 substrate binding pocket.*
5 Teplyakov AV, van der Laan JM, Lammers AA, Kelders H, Kalk KH, Misset O, Mulleners LJ, Dijkstra BW.
Protein Eng. 1992 Jul;5(5):413-20.
- 10 *Selection of a subtilisin-hyperproducing Bacillus in a highly structured environment by D. Naki, C. Paech, G. Ganshaw, V. Schellenberger. Appl Microbiol Biotechnol (1998) 49:290-294.*
- Requirements for transformation in Bacillus subtilis by Anagnostopoulos, C. and Spizizen, J. in J. Bacteriol. 81, 741-746 (1961).*
- 15 *Culture Medium for Enterobacteria by Frederick C. Neidhardt, Philip L. Bloch and David F. Smith in Journal of Bacteriology, Sept 1974. p736-747 Vol. 119. No. 3.*

20 **Table 1**

A1E						
A1D						
A1R						
A1K						
W6R						
G7N						
I8V						
R10C						
Q12H						
G23A						
F24S	L148G					
G25S						

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V26S						
V26S	N218S					
V26T						
E27R						
V28C						
V28S						
A29G						
V28T						
V30A						
L31A						
L31I						
L31T						
L31V						
R45I						
T38S						
G47D						
G47S						
S49D						
S49E						
D60N						
G61E						
G61K						
G61R						
G65M						
T66D						
T66E						
G69G	Q12R					
I72C						
I72L						
I72V						
A73L						
A73G						
A73T						

- 25 -

A73V						
L82R						
A85D						
A85G						
A85L						
A85S						
A85V						
A85Y						
P86E						
P86H	E271G					
P86D						
P86Y						
A85G						
A88S						
L90A						
L90I						
L90M						
L90V	N204D					
A92E						
A92R						
V93A	S103C					
V93I						
V93G						
V93S						
V93T	E136G					
K94T						
K94Q						
G97C						
G97E						
S99C						
S99D						
S99G						
S103D						

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S103E						
S103T						
S105D						
S105E						
S105G						
S105R						
W113D						
A114C						
A114G						
A114S						
A114T						
N116D						
N117S						
M119A						
M119C						
M119F						
M119G						
M119S						
M119T						
M119V						
Q121i						
H120R						
G127A						
S128D						
S128L						
E136R						
V139A	V150A					
A142E	E89G					
V147C						
V147G						
V147L						
V147S						
L148G						

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L148G	F24S					
L148W						
V149A						
V149F						
V149G						
V149H						
V149S	Q12H					
V149W						
V150A	T38S					
V150C	N218S					
V150F						
V150L						
A151V						
S156E						
S156D						
A169G						
R170M						
A174G	N204D					
A174S	G52E	A172T				
A174S						
A174T						
G178C	N43D					
G178L						
G178S						
I198A						
I198L						
I198M	V93I					
I198V						
I198V	V30A					
I198T						
M199V						
A200S	N204D					
P201C						

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P201S	L50F					
P201S	T57A					
V203R						
V203D						
V203E						
V203L						
V203S						
S216D						
S216E						
S216R						
N218S						
A231G	M119V					
A231S						
A232C						
A231V	Q206R					
A232G						
A232I	A108V					
A232L						
A232M						
A231V	Q206R					
A232N	N116D					
A232N	I16D					
A232T						
A232V						
A232S						
L233G						
L233V						
I246M						
I246V						
R247C						
S256G						
T253D						
T253E						

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T253K						
T253R						
G258D						
G258E						
G258K						
G258R						
G264S	R145G					
L267I	Y263H					
L267R						
L267R	S99G					
L267R	N252S					
A270L						
A270V	E136R					
A273S						
T260A						

Example 5

A large number of the protease variants produced in Example 1 were tested for performance in two types of detergent and wash conditions using a microswatch assay described in "An improved method of assaying for a preferred enzyme and/or preferred detergent composition", U.S. Serial No. 09/554,992 [WO 99/34011].

Table 2 lists the variant proteases assayed and the results of testing in two different detergents. For column B, the detergent was 7.6 g/l filtered Ariel Regular (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.5 ppm enzyme was used in each well at 40°C [European conditions]. For columns A, the detergent was 0.67 g/l filtered Tide Opal (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.5 ppm enzyme was used in each well at 20°C [Japanese conditions]. A performance index was calculated by the following formula :

Cleaning performance of the variant divided by cleaning performance of GG36 (wild-type)

Four performance values were averaged to arrive at the values shown in Table 2.

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Table 2

				A	B	
GG36 ¹				1.00	1.00	
GG36-	L31A				1.4	
GG36-	L82R				1.2	
GG36-	V203R				1.6	
GG36-	L233G				1.2	
GG36-	G258R				1.6	
GG36-	L267R				1.2	
GG36-	A270L				1.3	
GG36-	L31I			1.3		
GG36-	L31V			1.4		
GG36-	A85G			1.5		
GG36-	P86D			1.2		
GG36-	A92E			1.6		
GG36-	L148G			1.5		
GG36-	V149W			1.4		
GG36-	A151V			1.3		
GG36-	P201C			1.3		
GG36-	V203E			1.5		
GG36-	F24S	L148G			1.2	
GG36-	L50F	P201S			1.2	
GG36-	S99G	L267R			1.2	
GG36-	T57A	P201S			1.1	
GG36-	Q206R	A231V			1.3	
GG36-	N252S	L267R			1.2	
GG36-	Q136R	A270V			1.4	
GG36-	L90V	N204D		1.4		
GG36-	A172T	A174S	G52E	1.1		
GG36-	A174G	N204D		1.2		
GG36-	A200S	N204D		1.2		
GG36-	R145G	G264S		1.1		

¹GG 36 is the wild type protease of *Bacillus lentus* (SEQ ID NO. 4)

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As a result of the above described assays, some variants exhibited a performance index greater than that of the GG36 wild type protease. For example, the variants L31A, L82R, V203R, L233G, G258R, L267R, and A270L exhibited performance indices of 1.4, 1.2, 1.6, 1.2, 1.6, 1.2, and 1.3 respectively (Column B), in a microswatch assay (WO 99/34011) under European conditions (15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, 40 degrees Centigrade, 0.5 ppm). For example, the variants L148G-F24S, P201S-L50F, L267R-S99G, P201S-T57A, A231V-Q206R, L267R-N252S, and A270V-Q136R exhibited performance indices of 1.2, 1.2, 1.2, 1.1, 1.3, 1.2, and 1.4 respectively (Column B), in a microswatch assay (WO 99/34011) under European conditions (15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, 40 degrees Centigrade, 0.5 ppm). The variants L31I, L31V, A85G, A92E, L148G, V149W, A151V, P201C and V203E exhibited performance indices of 1.3, 1.4, 1.5, 1.2, 1.6, 1.5, 1.4, 1.3, 1.3, and 1.5 respectively (Column A), in the Microswatch 96 microtiter well plate (WO 99/34011) assay under Japanese conditions (3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness 20 degrees centigrade, 0.5 ppm). The variants N204D-L90V, A174S-A172T-G52E, A174G-N204D, A200S-N204D, R145G-G264S exhibited performance indices of 1.4, 1.1, 1.2, 1.2 and 1.1 respectively (Column A), in the Microswatch 96 microtiter well plate (WO 99/34011) assay under Japanese conditions (3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness 20 degrees centigrade, 0.5 ppm).

Example 6

An additional number of the protease variants produced in Example 1 were tested for performance in two types of detergent and wash conditions using a microswatch assay described in "An improved method of assaying for a preferred enzyme and/or preferred detergent composition", U.S. Serial No. 09/554,992 [WO 99/34011].

Table 3 lists the variant proteases assayed and the results of testing in three different detergents. For column A, the detergent was 7.66 g/l filtered Ariel Regular (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme was used in each well at 40°C. For column B, the detergent was 4.7 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.3 ppm enzyme was used in each well at 40°C. For column C, the detergent was 1.00 g/l filtered Tide Opal (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 6 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 0.5 ppm enzyme was used in each well at 20°C. For column D, the detergent was 0.66 g/l filtered Tide Opal (Procter & Gamble,

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Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness, and 1.0 ppm enzyme was used in each well at 20°C [Japanese conditions].

Table 3

				Ariel Regular	Ariel Futur	NA	Japa nese
GG36				1.00	1.00	1.00	1.00
A1E				0.51	0.51	1.15	1.92
A1D				0.76	0.68	1.04	1.5
A1R				1.51	1.47	0.25	0.17
A1K				1.39	1.28	.43	0.45
G47D				0.16	0.04	0.65	2.45
G61E				0.68	0.59	1.30	2.62
G61K				1.08	1.16	0.58	0.27
G61R				1.72	1.53	0.25	0.09
T66E				0.43	0.02	0.69	3.66
T66D				0.01	0.01	1.15	2.70
P86E				1.28	0.75	1.01	1.25
A92R				0.98	1.26	0.6	0.82
S105R				1.57	1.11	0.18	0.16
S105E				0.17	0.25	1.63	3.33
S105D				0.36	0.29	1.57	2.55
W113D				0.54	0.55	1.05	2.03
V203D				0.34	0.71	1.57	2.96
V203E				0.40	0.72	1.62	2.95
V203R				1.4	0.89	0.12	0.29
S216R				1.75	1.19	0.15	0.13
S216E				0.49	0.70	1.43	3.06
S216D				0.48	0.17	1.11	2.20
T253D				0.65	0.48	0.96	1.23
T253E				0.81	0.67	1.09	1.3
T253K				1.34	1.19	.74	.5
T253R				1.6	1.7	0.66	0.48

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G258E				0.69	0.83	1.21	1.85
G258D				0.94	0.82	1.12	1.95
G258R				1.9	1.4	0.34	0.38
G258K				1.54	1.18	0.50	0.47

As shown in Table 3 above, several variants displayed increased wash performance under "Japanese conditions" as compared to the GG36 wild-type; some variants displayed increased wash performance under "European conditions" [Ariel and Futur] as compared to the GG36 wild-type; several variants displayed increased wash performance under "North American" conditions. Several variants displayed increased wash performance under more than one wash condition, e.g., North American and Japanese conditions.

Example 7 Thermostability

Thermal stability of protease variants in European detergent solution was examined.

Materials :

iEMS Incubator (Lab systems)

Microtiter plate Reader

ASYS Multispense

Beckman Biomek FX robot

96-well microtiter plate

Ariel Futur detergent (batch'97)

N-Succinyl-Ala-Ala-Pro-Phe p NitroAnilide(AAPF); Sigma S-7388

Tween 80; Sigma P-8074

Tris(hydroxymethyl)aminomethane (Tris); T-1378

Sample preparation:

Enzyme samples were diluted to about 6.0 ppm (protein) starting concentration in 10 mM NaCl/0.005% Tween 80®. A 10 µl of diluted enzyme solution was transferred into 190 µl of unfiltered 3.4 g/L Ariel Futur (Procter & Gamble, Cincinnati, Ohio, USA) with 15 grains per gallon water hardness. The pH was adjusted to 8.6.

Samples were assayed using standard succinyl-ala-ala-pro-phe-para-nitro anilide ("SAAPFpNA") assay (Delmar, E.G., et al Anal. Biochem. 94 (1979) 316-320; Achtstetter,

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Arch. Biochem. Biophys 207:445-54 (1981)) (pH 8.6, ambient temperature) prior to incubation. For the assay, 10 ul of the sample solution and 200 ul of 1mg/ml SAAPFpNA substrate in 100mMTris pH 8.6 [and 0.005% Tween-80]. After standing at room temperature for thirty minutes after mixing, the absorbance at 405 nm (OD₄₀₅) was

5 determined. The samples were then incubated at 55 C for 20 minutes and the absorbance at 405 nm (OD₄₀₅) was determined. The remaining activity was calculated by dividing the OD₄₀₅ before incubation with the OD₄₀₅ after incubation. Column A depicts the residual activity of the variant divided by the residual activity of the wild-type GG36. For clarification, the mutants were made in GG36, e.g., G7N means the glycine at position 7 was substituted

10 with an asparagines. The results are depicted in Table 4.

Table 4

	A					
GG36	1.0					
G7N	1.8					
I8V	1.1					
G23A	1.2					
V26T	1.3					
V28C	1.2					
V28S	1.3					
A29G	1.7					
V30A	1.7					
L31A	1.4					
L31T	1.9					
G65M	1.3					
N117S	1.4					
I72C	1.5					
I72L	1.3					
I72V	1.2					
A73G	1.2					
A73T	1.4					
A85G	1.1					
A85S	1.5					
A85V	1.1					

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P86Y		1.3					
A88S		1.5					
L90A		1.2					
L90I		1.4					
L90M		1.5					
V93G		1.2					
V93S		2.5					
A114C		1.1					
A114G		1.2					
A114S		1.2					
A114T		1.2					
M119A		1.5					
M119C		1.1					
M119F		1.4					
M119G		1.2					
M119Q		1.2					
M119S		1.3					
M119T		1.1					
M119V		1.1					
M119L		1.5					
V147C		1.2					
V147G		1.1					
V147S		1.1					
V147L		1.1					
L148G		1.9					
V149A		1.3					
V149F		1.3					
V149G		1.4					
V149H		1.4					
V150F		1.2					
V150L		1.2					
V177R		1.3					
G178L		2.7					

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G178S		2.0					
I198A		1.1					
I198L		1.3					
I198T		1.2					
I198V		1.4					
V203A		1.5					
V203T		1.3					
A228G		1.5					
A228R		1.1					
A228S		1.5					
A231S		1.3					
A232C		1.3					
A232G		1.2					
A232L		1.2					
A232M		1.3					
A232S		1.2					
A232T		1.2					
A232V		1.2					
I246M		1.3					
I246V		1.2					
A273S		1.1					
V26S	N218S	1.4					
V93T	E136G	1.4					
V139A	V150A	1.4					
E89G	A142E	1.3					
Q12H	V149S	1.5					
V150C	N218S	1.3					
T38S	V150A	1.3					
N43D	G178C	2.0					
V93I	I198M	1.2					
V30A	I198V	1.2					
M199V	A231G	1.1					
A108V	A232I	1.2					

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N116D	A232M	1.4					
Y263H	L267I	1.1					
V93A	S103C	1.4					

As a result of the thermostability studies , the variants set forth in Table 4 were found to exhibit thermostability under the above test conditions as compared to the wild-type GG36 protease.

5 Although the present invention has been discussed and exemplified in connection with various specific embodiments thereof, this is not to be construed as a limitation to the applicability and scope of the disclosure, which extends to all combinations and subcombinations of features mentioned and described in the foregoing as well as the attached claims.

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